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Carbonic anhydrase inhibitors: Design of spin-labeled sulfonamides incorporating TEMPO moieties as probes for cytosolic or transmembrane isozymes

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ABSTRACT

A series of spin-labeled sulfonamides incorporating TEMPO moieties were synthesized by a procedure involving the formation of a thiourea functionality between the benzenesulfonamide and free radical fragment of the molecules. The new compounds were tested as inhibitors of the metalloenzyme carbonic anhydrase (CA, EC 4.2.1.1) and showed efficient inhibition of the physiologically relevant isozymes hCA II and hCA IX (hCA IX being predominantly found in tumors) and moderate to weak inhibitory activity against hCA I. Some derivatives were also selective for inhibiting the tumor-associated isoform over the cytosolic one CA II, and presented significant changes in their ESR signals when complexed to the enzyme active site, being interesting candidates for the investigation of hypoxic tumors overexpressing CA IX by ESR techniques, as well as for imaging/treatment purposes.

In previous work from this laboratory,[1,2] we reported that fluorescein-labeled sulfonamides act as very potent inhibitors of the zinc enzyme carbonic anhydrase (CA, EC 4.2.1.1).[3] These fluorescein derivatives are now in early phases of development for applications as diagnostic tools and therapeutic agents for tumors overexpressing CA isozymes IX and XII (among the 16 presently known in mammals).[3–5] With the aid of such fluorescent inhibitors it was possible to prove the involvement of CA IX in tumor acidification processes which lead to an excessive production of H^+ ions in the extracellular space where the active site of CA IX and XII are situated,[4,5] and the possibility to reverse this deleterious phenomenon by blocking the enzyme activity.[1–5] The success of this approach has encouraged us to now investigate alternative possibilities for the labeling of CAs present in tumors (i.e., CA IX and XII) or related isoforms, eventually present in the cytosol (CA I, II, VII, and XIII) or mitochondria (CA VA and VB) of eukaryotic cells.[3] Introduction of a spin label in the molecules of sulfonamides acting as well-known inhibitors of these enzymes[3] seemed to us a straightforward continuation of our previous work.[1,2] In this letter we report the synthesis, CA inhibitory activity against some physiologically relevant isoforms (CA I and II, cytosolic isozymes, and CA IX, transmembrane, tumor-associated isoform)[3] as well as the ESR properties for a series of benzenesulfonamides incorporating 2,2,6,6-tetramethylpiperidinyl-1-oxyl (TEMPO) and thioureido moieties.

Spin-labeled sulfonamides were in fact reported in the 70s,[6] when little was known on the binding of sulfonamides within the CA active site, as no X-ray crystallographic structures of any isoform alone or in complex with inhibitors were available at that time. Some of the compounds **A–E** reported earlier[6–8] incorporated either five-membered (pyrrolidine-*N*-oxide) or six-membered (piperidine-*N*-oxide) radical moieties in their molecule, together with the classical benzenesulfonamide warheads known to coordinate (in deprotonated form, as sulfonamidate anion) to the Zn(II) ion present in the enzyme active site and crucial for its catalytic activity (Chart 1).[3] These compounds were basically used for gaining insight on the topology of the CA active site and no detailed inhibition studies are available with them, also because only isoforms I and II were known in the period when they were reported.[6] Furthermore, the possibility of exploiting the ESR signals of these earlier compounds of types **A–E** for imaging/treatment purposes has never been taken into account. There is in fact a stringent need

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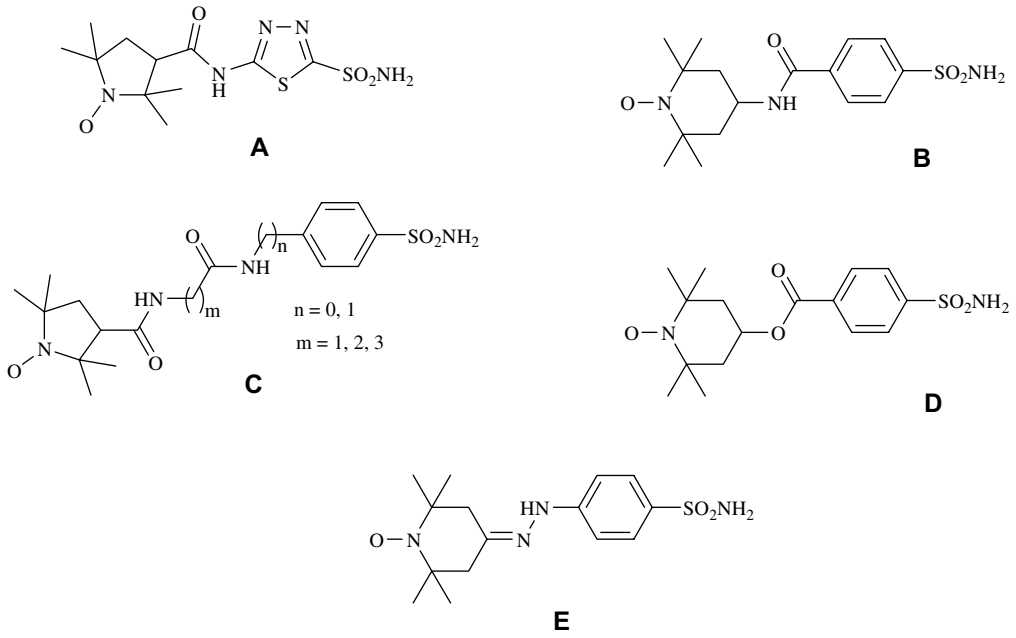

Chart 1. Spin-labeled sulfonamides **A–E** reported earlier.

of CAIs which can be used for the selective labeling of CA isozymes involved in various pathological processes,[3,7,8] as exemplified among others also by the very recent report of Christianson's group of a ^{129}Xe-cryptophane-sulfonamide biosensor complexed to hCA II which can be used as a diagnostic tool for ^{129}Xe-magnetic resonance imaging (MRI).[8]

The design of the new CA inhibitors (CAIs) **1–10** reported here was approached by the classical tail strategy,[2,3] that is, maintaining the benzenesulfonamide head present in the spin-labeled compounds **A–E**[6] reported earlier, and by incorporation of a thiourea linker between this and the free radical TEMPO tail (Scheme 1), which was not present in any of the earlier derivatives. The benzenesulfonamide moiety was chosen due to its ability to tightly bind to the zinc ion within the enzyme active site, as shown by extensive X-ray crystallographic work from this and other groups.[1–3,8–11] Furthermore, the phenyl ring belonging to the benezenesulfonamide moiety may be substituted in positions 3 and 4 (with respect to the sulfamoyl group) with halogen atoms, as some of these derivatives were shown earlier to lead to potent CAIs.[1–3] The central thiourea linker motif was chosen because it was shown earlier that compounds incorporating it act as potent hCA I, II, and IX inhibitors.[12,13] We then employed the 2,2,6,6-tetramethylpiperidine-1-oxyl (TEMPO) moiety as the tail group. This free radical moiety is active in the ESR experiments, and possesses a simple spectrum.[14] In addition, it is stable, it induces a relatively good water solubility to the compounds incorporating it, and its spectral features (such as line width and intensity) can be regulated by tissue oxygen or redox status in in vivo experiments. This latter property also renders newly synthesized molecules containing this radical scaffold to be incorporated in spin probes that interact with biomolecules such as enzymes.[14,15]

The compounds **1–10** were prepared as shown in Scheme 1 by employing two alternative but similar reaction methods (depending on the stability of the isothiocyanate intermediates). The first method consisted in the reaction of aminosulfonamides with thiophosgene in acidic medium which leads to the corresponding isothiocyanate-sulfonamides.[12,13] Reaction of these intermediates with amino-TEMPO in acetonitrile led to thioureas **1** and **4–10**. As the precursor sulfonamide isothiocyanates could not be prepared in acceptable yields for the subsequent synthesis of compounds **2** and **3**, an alternative method was employed, that is, reaction of TEMPO isothiocyanate (obtained from amino-TEMPO and $CSCl_2$ similar to what is described above)[12,13] with the corresponding amines (Scheme 1).[16,17]

The inhibition data of the new sulfonamides **1–10** and standard, clinically used inhibitors such as acetazolamide **AAZ**, methazolamide **MZA**, ethoxzolamide **EZA**, dichlorophenamide **DCP**, and indisulam **IND** (Chart 2) against the cytosolic isozymes hCA I, hCA II, and the transmembrane tumor-associated isozyme hCA IX (h = human isoform) are presented in Table 1.[18] The following structure–activity relationships (SARs) should be noted: (i) against the ubiquitous, house-keeping, and physiologically relevant isoform hCA II the new sulfonamides **1–10** showed good inhibitory activity, with inhibition constants (K_i values) in the range of 12–165 nM. The derivatives with a *meta* substitution pattern on the benzenesulfonamide ring showed the least efficient inhibitory activity (K_is of 165 and 152 nM, respectively, for derivatives **4** and **7**). Compounds **1–3** showed a compact behavior of efficient CAIs, with K_is of 28–42 nM. An increased affinity for hCA II has been observed for the halogeno-substituted derivatives **5** and **6**, with the chlorine-substituted benzenesulfonamide **6** being a very efficient CAI, comparable to the clinically used compounds **AAZ, MZA**, and **EZA** (Table 1). The sulfanilyl-sulfonamides **8–10** were slightly less efficient hCA II inhibitors as compared to **6**, but they appreciably inhibited the enzyme with inhibition constants in the range of 20–47 nM. Thus, we evidenced various types of substitution patterns of the TEMPO-containing CAIs that lead to efficient, low nanomolar inhibitors of the physiologically relevant isozyme hCA II; (ii) the inhibition data of compounds **1–10** against the tumor-associated isozyme hCA IX (our target for imaging or treatment purposes)[1] showed them to possess excellent inhibitory activity, with K_is values in the range of 7–220 nM (Table 1). Similar to the hCA II inhibition discussed above, the *meta* substitution pattern present in derivatives **4** and **7** led to the least effective inhibitory properties (K_is of 132 and 220 nM, respectively) for the corresponding sulfonamides. The other compounds reported here were much better hCA IX inhibitors as compared to **4** and **7**, with inhibition constants in the range of 7–41 nM. SAR was rather sim-